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Transcriptional Expression of Epstein-Barr Virus Genes and Proto-Oncogenes in North African Nasopharyngeal Carcinoma

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Cases of nasopharyngeal carcinoma (NPC) from North Africa show an unusual bimodal age distribution. As elsewhere, the tumor is closely associated with the presence of Epstein-Barr virus (EBV). The expression of EBV genes and c-onc genes was studied in biopsy specimens from tumors at different clinical stages from 11 young (10 to 30-year-old) and 11 adult (30 to 65-year-old) patients. It was found that the two age groups do not differ in their pattern of gene expression, that there is a tendency for later stage biopsies to express more viral and c-onc transcripts, and that samples expressing larger numbers of EBV genes also tend to express many different c-onc specificities.

KEY WORDS: NPC, EBV, proto-oncogenes, transcription, tumour progression

INTRODUCTION

Nasopharyngeal carcinoma (NPC) is a significant public health problem in Southern China, much of South Asia and North Africa. The age distribution is unusual in North Africa in that a peak of incidence occurs among young subjects (10–30 years) as well as the more usual occurrence in adults (30–65 years). As in other countries, North African NPC is closely associated with Epstein-Barr virus (EBV), whose genome is generally present in tumor biopsies [Bouzid et al., 1994], and most patients have high antibody titers to EBV antigens [Ablashi et al., 1981]. A study of viral polymorphism showed no significant difference between young and adult North African NPC patients [Bouzid et al., 1994], but only some 50% of young patients possessed IgA antibodies to viral capsid antigens (VCA) antigens despite a vigorous IgG response [Bouguermouh, 1985].

EBV infection of NPC biopsy specimens may be of either the productive (lytic) or latent type [Raab-Traub et al., 1983], and involves differential expression of viral genes. Transcription of BARF0 has been studied extensively by Northern hybridization [Gilligan et al., 1991; Chen et al., 1992; Karran et al., 1992], but that of other genes such as LMP1, LMP2, EBNA1, EBERs and BLZF1 has only been demonstrated in NPC biopsies by reverse transcriptase-polymerase chain reaction (RT-PCR) [Brooks et al., 1992; Busson et al., 1992] or in situ hybridization [Cochet et al., 1993; Wu et al., 1991]. Viral peptides involved in latent infection, such as EBNA1 and LMP1, are expressed by a variable number of cells in NPC biopsies [Fahraeus et al., 1988; Young et al., 1988], and early proteins such as ribonucleotide reductase, EA-D (BMRF1), EA-R (BHRF1) and BZLF1 have been detected using monoclonal antibodies [Luka et al., 1988; Lung et al., 1989; Cochet et al., 1993]. However, the production of significant lytic cycle proteins as exemplified by EA-D in such samples was not confirmed by another study [Young et al., 1988].

Cellular oncogene expression is probably involved in the etiology of NPC, but only bc12, ras and c-myc have been studied to date [Lu et al., 1993; Porter et al., 1994]. We have therefore studied the expression of a panel of EBV genes and of cellular oncogenes involved in solid tumor formation, such as the Ras family [Barbacid, 1987] or in EBV infection, such as c-fgr and c-myc [Lombardi et al., 1987; Brickell and Patel, 1988] in biopsies from young and adult North African NPC patients. Southern hybridization of ^{32}P-labelled cDNA to RNA from NPC biopsies was used since this technique has been shown to detect abundant transcripts from several different genes in small samples of tumor tissues [Raab-Traub et al., 1983; Zhang et al., 1992]. A total of 20 genes using 22 biopsy samples were tested. We show that LMP2 and certain early EBV genes are generally expressed in biopsies from patients of all ages, and that proto-oncogene expression appears to increase with tumor stage progression.

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MATERIALS AND METHODS

Clinical Samples

Biopsy specimens (0.2–0.5 g) were obtained from 11 young (<30 years old) or older (>30 years old) Algerian or Tunisian NPC patients, and snap frozen immediately in liquid nitrogen and stored at −70°C. Tumors were classified as undifferentiated (WHO stage III); 19 specimens, poorly differentiated (WHO stage II); 2 specimens or well differentiated (WHO stage I); 1 specimen by histological examination undertaken by Dr. Bouhadref (Kouba hospital, Algeria) or Dr. Chouiter (Pierre & Marie Curie Center, Algeria). Clinical staging of the patients was carried out according to the International Union Against Cancer protocol [Spiesel et al., 1982] with tumor involvement increasing from T1 to T4, and adenopathy increasing from N1 to N4. Serum from each patient was stored at −20°C.

Cell Cultures

BJAB, IB4, B95-8 and P3HR1 cells were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum and antibiotics; Raji cells were treated in addition with 13-O-tetradecanoyl-phorbol ester (50 ng/ml) and sodium butyrate (2 mM; TPA-SB) as described previously [Ooka et al., 1983]. EBV-negative human epithelial cells RHEK-1 [Rhim, 1989], generously donated by Dr. Rhim, National Cancer Institute, and HaCaT [Boukamp et al., 1988], were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum.

Serology

IgA antibodies to EBV EA and VCA antigens were titrated by indirect immunofluorescence on Raji and P3HR1 cells, respectively [Henle et al., 1970].

Hybridization Fragments From EBV and Cellular Proto-Oncogenes

Expression of the EBV genes EBNA1, EBNA2, LMP1, LMP2, BARF0 (latency), BALF2, BALF5, BARF1, BZLF1 (early) and BALF4 (late) were detected using specific recognition fragments as follows: EBNA1: 1.1 Kb *Hinf I* fragment from *BamH* 1-K; EBNA2: 0.75 Kb BamHI-HinfI fragment from *BamH* 1-H; LMP1: 1.93 Kb *Xho I* fragment from *Eco*R1-D; LMP2: plasmid p1051-2 (Dr. Cho, Berkley, CA); BARF0: 1.1 Kb spliced sequence from genome positions 157117 to 160984 [Zhang et al., 1993]; BALF2 and BALF4: pZip-Neo plasmids containing sequences coding for the 135 kDa DNA binding protein and for gp110, respectively [Gong et al., 1987]; BALF5: *sphI* fragment from genome positions 153171 to 157114; BARF1: a 0.74 Kb c-DNA sequence [Wei and Ooka, 1989]; and BZLF1: plasmid pSV*NaeIBamZ* (Dr. Kieff, Harvard Medical School, Boston).

Expression of cellular proto-oncogenes was evaluated using sequences from plasmids capable of detecting gene expression in human samples. The plasmid pMycEc1 contains the third exon of human c-myc; pCKFos (Dr. R. Muller, EMBL, Heidelberg, Germany) contains chicken c-fos, p-fgr (Dr. Tamura, Institut für Virologie, Liebig Universität, Germany) detects c-fgr, pE7 (Dr. Y.-H. Xu NCI, Bethesda, MD) detects c-erb-B, Fsrc (Dr. J. Mulin, Harvard Medical School, Boston) contains feline c-src, Ki-ras and Ha-ras are detected by pHB11 and pKBE2, respectively (Dr. Lowry, NCI, Bethesda, MD), N-ras is detected by either p6a1 (c-DNA) or pAT153 (genomic) [Taparowsky et al., 1983], pPDGF-A (Dr. C. Betzholtz, University Hospital, Uppsala, Sweden) detects PGDF and FGF is detected by a commercial construct (California Biotechnology Inc., Mountain View, CA).

Nucleic Acid Extractions

The frozen biopsy samples were divided into two, and the DNA was extracted from half as described previously [Ooka and Calender, 1980], and the RNA from the other half using a commercial kit (Stratagene, California, USA).

Estimation of Viral DNA

A rough estimated of the average number of EBV genomes per cell in the NPC biopsy samples was obtained by dot blot analysis as previously described [Zhang et al., 1992; Bouzid et al., 1993]. Briefly, 10 μg of DNA from each specimen was linearized by digestion with *Bam*H1, spotted onto hybond N^+ filters (Amersham, UK) and hybridized with labelled *Bam*H1-M EBV fragment (4696 bp) as a probe. The retained radioactivity was compared with a standard curve obtained by diluting DNA from Raji TK^+ cells, which have 60 EBV genome copies/cell [Ooka et al., 1986], with varying amounts of DNA from EBV-negative BJAB cells.

Preparation of Radioactive Single-Stranded cDNA

Total cellular RNA (50–100 μg) from the biopsies was reverse-transcribed with 200 units of reverse transcriptase (M-MLV-H-RT-Superscript; BRL-GIBCO, France) in a solution containing 50 mM Tris (pH 8.2), 10 mM $MgCl_2$, 75 mM KCl, 10 mM Dithiothreitol, 1 mM each of dATP, dGTP and dTTP, 10 nM dCTP, 0.5 μg oligo-dT15, 2 units/M1 RNase inhibitor and 100 μCi (^{32}P)dCTP at 42°C for 60 min. The reaction was then stopped by adding EDTA and SDS to final concentrations of 0.2 and 0.25 M, respectively. Free nucleotides were removed by passage through a Sephadex G50 column (Pharmacia, Sweden).

Southern Blotting

DNAs from the EBV or cellular proto-oncogene sequences were freed by digestion with the relevant restriction enzymes, separated by electrophoresis through 1% agarose gels and blotted onto reinforced nitrocellulose membranes (Schleicher & Schuell, Germany), since nylon filters tended to give high backgrounds. The ^{32}P-labelled cDNAs (0.5×10^6 cpm) were hybridized to the filters for 18 hr at 65°C in the modified solution described by Ausubel et al. [1987] to improve reproducibility (50 mM Sodium phosphate pH 7.2; 7% SDS; 1% bovine serum albumin and 100 μg/ml salmon sperm DNA). The filters were then washed three times for 15 min at 65°C in

double concentration sodium citrate solution (2 × SSC) containing 0.1% SDS, twice in 0.1 × SSC containing 0.1% SDS, then exposed to film for 5 days. Negative controls consisted of radioactive cDNAs from EBV-negative epithelial (RHEK-1 or HaCaT) or lymphoid (BJAB) cells, and radioactive cDNA from TPA-SB induced Raji cells served as a positive control.

Northern Blotting

Polyadenylated RNAs were extracted from the biopsies of 4 adult patients at clinical stages T1, T2, T3 and T4 using streptavidin substituted magnetic particles (PolyATract mRNA isolation system III, Promega, Madison, Wisconsin, USA). Samples of 5–10 μ of these RNAs were separated by electrophoresis through 1% agarose gels containing formamide [Wei et al., 1994], then transferred by capillarity to Hybond N^+ filters which were hybridized under standard conditions, [Zhang et al., 1988] to probes from LMP1 and actin sequences labeled using a random primer kit (Stratagene). BARF1 probes did not label satisfactorily under these conditions so a riboprobe was prepared from a BARF1 cDNA sequence in the pGEM vector using SP6 polymerase according to the manufacturer's recommendations (Promega, France). Filters were hybridized with this riboprobe overnight at 60°C in a solution containing 50% formamide, 0.05 M sodium phosphate (pH 7.2), 2.5 × Denhardt solution, 5 × SSC, 100 μg/ml salmon sperm DNA and 200 μg/ml yeast RNA, then washed twice for 30 min in 2 × SSC containing 0.1% SDS at 60°C and twice for 20 min 0.1 × SSC containing 0.1% SDS at 60°C.

Immunoblotting

Proteins were extracted from the 4 adult biopsies representing the T1 to T4 clinical stages by suspension in 10 vols of RIPA buffer (0.1% SDS, 0.5% Desoxycholate, 0.5% Triton X-100, 0.4 M NaCl, 5 mM EDTA and 20 mM Tris/HCl pH 7.6). After sonication, the protein concentration was estimated (Bio-Rad protein assay, Bio-Rad Laboratories, Inc.) and 30 to 50 μg of protein was diluted with an equal volume of gel sample buffer (10% glycerol, 5% β mercaptoethanol, 2% SDS, 0.05% Bromophenol blue, 50 mM Tris/HCl pH 6.8) and boiled. The proteins were then separated by electrophoresis through polyacrylamide gels, and blotted onto reinforced nitrocellulose [Rickinson et al., 1987]. Nonspecific sites on the filters were blocked by incubation for 1 hr in phosphate-buffered saline containing 0.1% Tween 20 and 5% skimmed milk. EBNA1 antigen was detected by a monoclonal antibody (kindly provided by Dr. E. Kieff, Harvard Medical School) and LMP1 by monoclonal S12 (kindly provided by Dr. Thorley-Lawson, Tufts University, Boston, Massachusetts, USA) both used at 1:1,000 dilution. After incubation with antibody for 1 hr at room temperature, the filters were washed, and the fixed antibodies revealed by incubation with peroxidase-labelled anti-mouse Ig then visualized by an enhanced chemioluminescence system (ECL, Amersham, England).

RESULTS

EBV Serology and Viral Genome Copy Number

The most striking differences between young (<30 years old) and older (>30 years old) North African NPC patients concern the average copy number of EBV genomes per cell in the tumor, and the level of the IgA response to EBV antigens. Only 2 of 11 young patients had appreciable IgA antibodies to either early or late EBV antigens, whereas all except one of 11 adult patients had significant IgA titers to at least one antigen. Similarly, only one of the 11 young patients had more than 1 or 2 copies of EBV genome per tumor cell, whereas 7 of the 11 adult patients had high copy numbers (Table I). Conversely, there appears to be little relation between either of these parameters and the clinical staging of the disease.

Expression of EBV Genes in Tumor Biopsies From Young and Adult Patients

Abundant expression of viral genes as detected by cDNA Southern blotting is rather irregular, though there is some tendency towards a larger number of transcripts in biopsies from patients with more severe clinical disease (Table II). Some latency-associated gene transcripts are often observed; LMP2 is expressed in the majority of samples from all clinical stages while BARF0 and EBNA1 expression appears more often in more severe disease, but abundant EBNA2 and LMP1 transcripts were never observed. The expression of the early gene BALF5 appears to increase with disease stage; BZLF1 is observed in a small number of biopsies from all stages, and BALF2 occurred sporadically in intermediate stage specimens. BARF1 was never observed. The late gene transcript BALF4 was seen in a small proportion of advanced tumor biopsies. Expression of EBNA1, BZLF1 and BALF4 was rather more frequent in samples from young than from adult patients, but the numbers involved are small (Table II). Two tumor biopsies (A3 and A6) did not express detectable levels of any of the EBV-related mRNAs tested.

Control experiments never detected EBV-related transcripts from negative epithelial cell lines (Fig. 1A and B), while induced Raji cells were strongly positive for EBNA1, LMP2, BARF0, BALF2 and BALF4, weakly positive for LMP1 and BALF5 and negative for EBNA2, BZLF1 and BARF1 (Fig. 1C). The lack of detection of BZLF1 mRNA in induced Raji cells may be due to linkage of its transcription to EA expression; we observed significant transcripts in Raji cultures where more than 20% of the cells expressed EA (data not shown). The weak expression of LMP1 mRNA in Raji cells was surprising, and we confirmed the presence of the corresponding peptide in these cells and in biopsy cells from all 4 clinical stages of NPC, none of which had detectable mRNA (Fig. 3a), by immunoblotting (Fig. 2b). In agreement with previous studies, all these cells expressed both LMP1 and EBNA1 (Fig. 2a and b) peptides. The molecular weight of LMP1 protein in all our tumor biopsies was around 65 kDa (Fig. 2b), while in IB4 or Raji it was

TABLE I. Analysis of Young and Adult NPC Patients*

Biopsy	Stage	Age	WHO classification	EBV serology IgA VCA	EBV serology IgA EA	EBV copy
Young patients (0–30 years)						
Y1	T0N0	19	III	40	<10	1–2
Y2	T1N2	26	III	10	10	1
Y3	T1N3	15	III	160	<10	1
Y4	T1N2	17	III	<10	<10	15
Y5	T2N2	18	I	<10	<10	1
Y6	T2N3	19	III	<10	<10	1
Y7	T2N3	14	III	<10	<10	1
Y8	T3N3	18	III	<10	<10	1
Y9	T3N3	16	III	<10	<10	1
Y10	T3N0	17	III	<10	<10	1
Y11	T4N4	16	III	<10	10	1
Adult patients (>30 years)						
A1	T1N0	62	TP[a]	<10	<10	1
A2	T1N0	47	III	160	40	3–4
A2	T1N3	60	III	40	640	35–40
A4	T2N3	50	III	640	280	1
A5	T2N3	47	III	160	640	5
A6	T2N3	37	III	40	40	1–2
A7	T3N0	40	III	40	10	4
A8	T3N0	44	III	160	160	25
A9	T3N1	40	II	640	160	1
A10	T4N3	48	II	160	40	25
A11	T4N0	48	III	640	640	10–15

*Clinical staging of the patients was performed according to the International Union Against Cancer protocol with tumor involvement increasing from T1 to T4, and adenopathy increasing from N1 to N4 [Spiesel et al., 1982]. Type I: well differentiated; Type II: poorly differentiated; Type III: undifferentiated.
[a]TP: Tumor proliferation.

60–63 kDa as previously reported [Alfieri et al., 1991]. Similarly, the major EBNA1 polypeptide had a MW of 55–65 kDa in all biopsies, compared to 67 and 76 kDa in Raji and P3HR1, respectively (Fig. 2a). The BARF1 oncogene was expressed faintly in only one biopsy by the ^{32}P-cDNA method (not shown), but one pool of stage 3 biopsies gave a faint band of 1.1 Kb on Northern blot (Fig. 3b, lane 3), which could correspond to a mRNA transcribed from the BARF1 region [Zhang et al., 1988].

Expression of Cellular Proto-Oncogenes

The abundant expression of cellular oncogenes was also irregular in biopsy specimens (Table II). All cells tested expressed C-FOS, and the majority (13/22) of tumor biopsies also contained abundant c-myc transcripts. Transcripts of other oncogenes were present in 4/11 biopsies from young subjects, and 5/11 from adults. One adult specimen (A7) expressed only c-erb in addition to c-fos, but otherwise multiple expression of the supplementary c-onc genes was the rule. The multiplicity of c-onc expression correlated only weakly with the tumour stage, or with multiplicity of EBV-related gene expression. Six of 7 specimens positive for PDGF also expressed FGF, and all 6 samples which expressed K-ras were also positive for Ha-ras. Maximum c-onc expression was seen in biopsy A11 where abundant transcripts of 9 different oncogenes were observed.

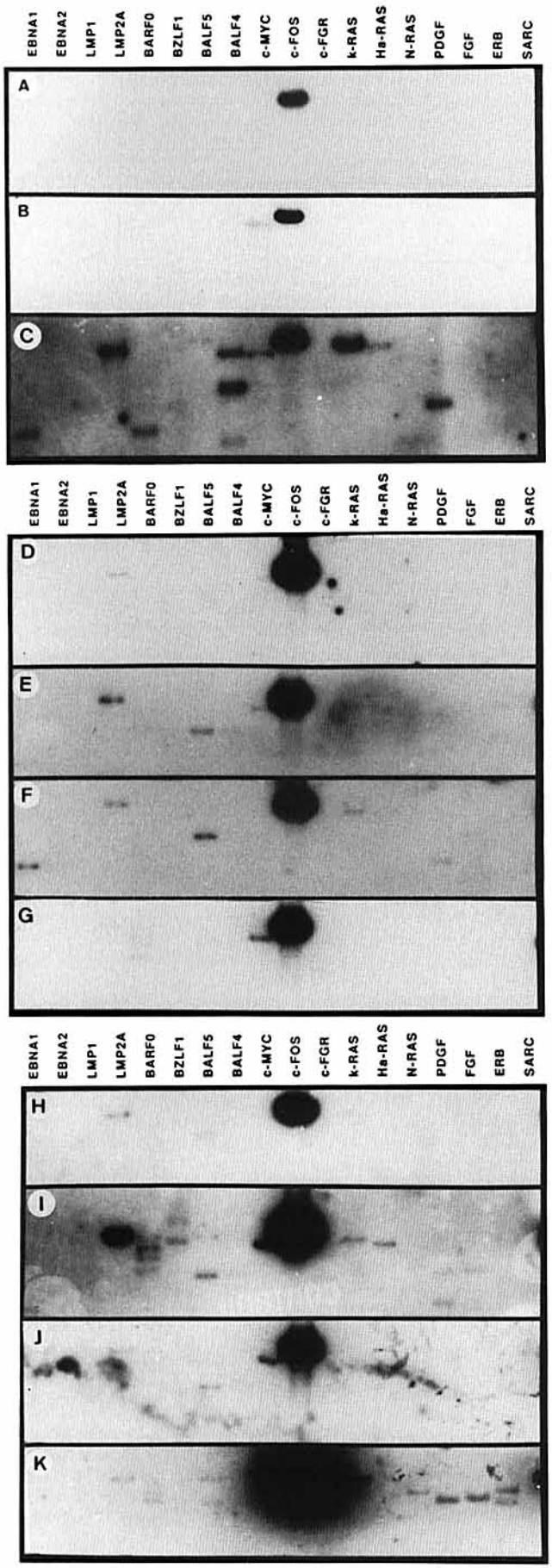
DISCUSSION

The transcriptional activity of some EBV genes and some cellular proto-oncogenes were investigated in NPC biopsies from North African patients. Southern blotting of ^{32}P-cDNA synthesized using cellular RNA as a template, followed by hybridization to specific probes, allows the detection of abundant transcripts, and can be used to examine several different activities in small samples. Previous observations, using RT-PCR [Brooks et al., 1992], that LMP2 is regularly transcribed in North African NPC biopsies, but that LMP1 and EBNA2 transcripts are not sufficiently abundant to be observed by Northern blotting [Gilligan et al., 1990] were confirmed. Despite this, LMP1 protein is present in biopsy specimens from all disease stages, whereas EBNA2 protein is never detected. These results are in accordance with those described previously [Young et al., 1988]. Other latency genes transcribed abundantly in some specimens are EBNA1, and BARF0. The early gene expressed most often is BALF5 which, like BARF0, tends to occur more regularly in cells from later stage disease. Late gene expression as typified by BALF4, was infrequent. Since the mRNAs coding for BALF5 (DNA polymerase) and BALF4 (gp 110) are in part transcribed from a region which codes for BARF0 on the opposite strand, it is possible that cross-hybridization could account for the signals observed. The three transcripts are not associated sys-

TABLE II. Expression of EBV Genes and Proto-Oncogenes According to Disease's Stage and Age of Patients*

		EBV GENES										C-ONCOGENES								
		Latent					Early				Late									
Stages	Biopsy	EBNA1	EBNA2	LMP1	LMP2	BARF0	BZLF1	BALF5	BALF2	BARF1	BALF4	c-Fos	c-Myc	PDGF	FGF	c-fgr	K-ras	Ha-ras	N-ras	ERB
	Young patients (0 to 30 years)																			
T_1	Y1				●			●				●								
	Y2				●	●						●		●	●	●				
	Y3				●			●				●	●							
	Y4				●		●					●	●							
T_2	Y5				●							●								
	Y6	●		●	●			●				●								
	Y7	●			●	●		●	●		●	●	●	●			●	●		●
T_3	Y8	●			●	●		●			●	●								
	Y9							●				●	●							
	Y10	●			●	●		●			●	●	●	●	●		●	●		●
T_4	Y11				●	●	●	●				●	●	●	●					
	Adult patients (>30 years)																			
T_1	A1				●							●	●							
	A2				●			●				●								
	A3											●	●							
T_2	A4	●			●	●	●	●	●		●	●	●	●	●		●	●		
	A5	●			●	●		●				●					●	●		
	A6											●								
T_3	A7				●							●								●
	A8				●	●		●				●	●							
	A9							●				●	●							
T_4	A10				●	●		●				●	●	●	●	●	●	●		●
	A11				●	●		●				●	●	●	●	●	●	●	●	●
	RHEK											●	●							
	HaCaT											●								

*Y1–11 correspond to biopsies from young patients and A1–11 from adult patients. Two EBV-negative human epithelial cells, RHEK-1 and HaCaT, were used as control cells. T1, Stage I; T2, Stage II; T3, Stage III; T4, Stage IV.
[a]Circles, positive transcription.



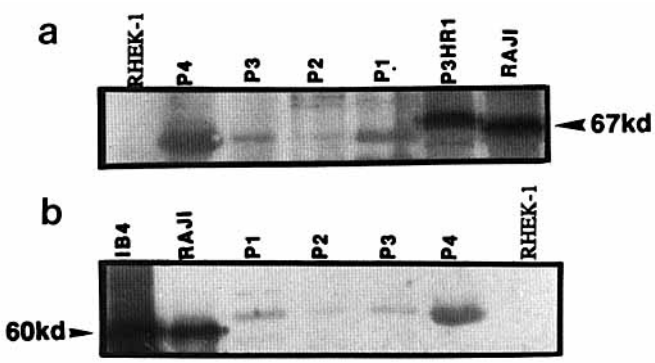


Fig. 2. Immunoblot of LMP1 and EBNA1 proteins in extracts from NPC biopsies. Protein extracts prepared from biopsies were loaded onto a sodium dodecyl sulfate-polyacrylamide (6% to 10%) gel. Extracts of EBV-negative RHEK-1 cells and EBV-positive cells (IB4, RAJI and P_3HR1) were also loaded as controls. Protein extracts were named as P: P1, A2 (Stage 1); P2, A4 (Stage 2); P3, A8 (Stage 3); P4, A11 (Stage 4). After electrophoresis, the proteins were transferred onto nitrocellulose and the nitrocellulose filters were contacted with anti-EBNA 1 **(a)** and anti-LMP1 S12 Mabs **(b).**

tematically in individual cell specimens; however, in view of the complexity of BARF0 transcription [Hitt et al., 1989; Gilligan et al., 1990; Chen et al., 1992; Karran et al., 1992; Zhang and Ooka, 1995], further studies using riboprobes are necessary to confirm our observations.

The transforming early gene BARF1 [Wei and Ooka, 1989; Wei et al., 1994] did not give rise to sufficiently abundant transcripts to be detectable by the ^{32}P-cDNA method, but weak bands were detected in 2 of 5 biopsies after Northern blotting and detection using a riboprobe. Transcripts of this gene were also difficult to detect in EBV-positive lymphoblastoid cell lines [Wei et al., 1994], so it is probably usually only transcribed at low levels. Few biopsies were found with abundant BZLF1 transcripts, though this gene has been detected by a sensitive RT-PCR method in NPC biopsies [Cochet et al., 1993], and other early genes were rarely transcribed abundantly. The expression of EA-D and EA-R in NPC biopsies has been reported [Luka et al., 1988; Lung et al., 1989], but generally early gene expression appears to be highly restricted in NPC as in EBV-induced lymphomas in the tamarin [Zhang et al., 1992].

The expression of cellular proto-oncogene in NPC tumor biopsies is again quite variable. All samples showed abundant transcription of c-fos, and many expressed c-

Fig. 1. Transcription pattern of NPC biopsies. ^{32}P-cDNAs used as probes were synthesized by mRNA extracted from EBV-negative normal epithelial cell lines, EBV-positive cell line and tumor biopsies of young and adult NPC patients at different stage of disease. The EBV genes and proto-oncogenes blotted onto nitrocellulose filters were hybridized for 18 hours and the filters were exposed for 5 days. **A:** ^{32}P-cDNA from EBV-negative RHEK-1 cell RNA. **B:** ^{32}P-cDNA from EBV-negative HaCaT cell RNA. **C:** ^{32}P-cDNA from TPA- and SB-treated EBV carrying RAJI cell RNA. **D–G:** Young patients (D, Y3; E, Y7; F, Y10; G, Y11); **H–K:** Adult patients (H, A2; I, A4; J, A8; K, A11). D, H: Stage 1 (T1); E, I: Stage 2 (T2); F, J: Stage 3 (T3); G, K: Stage 4 (T4). BARF0 and BZLF1 were not completely digested by *Eco* RI and *Bam* H1, respectively.

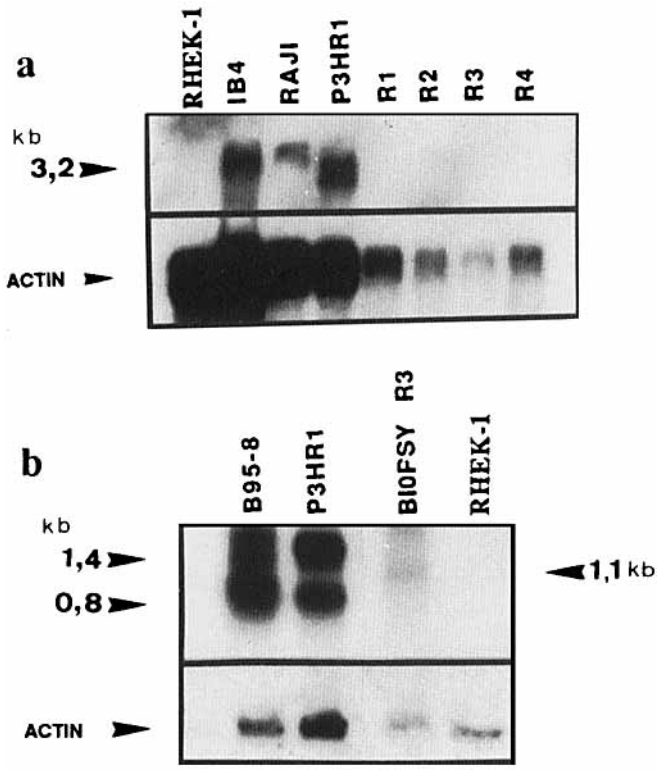


Fig. 3. Northern blot analysis of LMP1 **(a)** and BARF1 **(b)** transcripts from mRNA NPC biopsies. Each lane of a 1% agarose-2.2 M formaldehyde gel [Wei et al., 1994] contained about 5 μg of polyA$^+$ RNA from indicated cells and tumour biopsies. The molecular weights of RNA are indicated to the left of the figure by arrows. The lanes contain poly (A$^+$) cellular RNA from RHEK-1, IB4, RAJI, P3HR1 and B95-8 as controls. RNA from biopsies named as R: R1, Stage 1; R2, Stage 2; R3, Stage 3; R4, Stage 4.

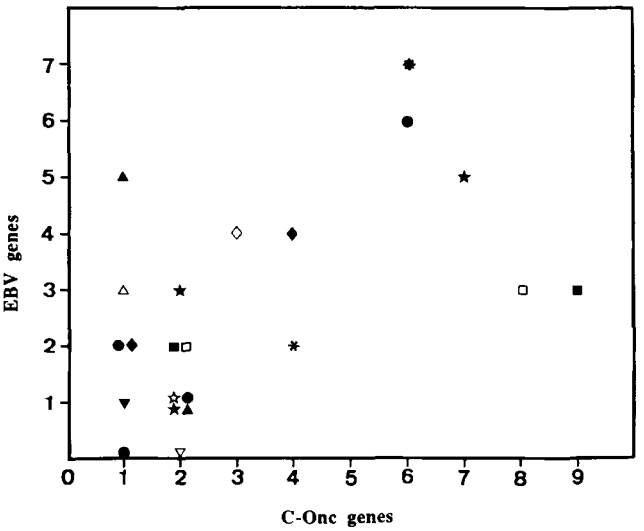


Fig. 4. Correlation between the number of C-ONC transcripts and the number of EBV transcripts in a given biopsy. Abscissa: the number of EBV genes. Ordinate: the number of c-onc genes. Each symbol corresponds to a biopsy.

myc and ras family genes which have been reported to be active in EBV-related head and neck squamous cell carcinoma [Field, 1992; Field et al., 1989] and in NPC [Porter et al., 1994]. There was some tendency for a larger number of c-onc transcripts to be present in samples from advanced disease, but we were unable to study successive samples from individual patients to confirm this observation since all NPC-positive subjects are given immediately chemotherapy or radiotherapy.

It has been suggested that the expression of EBV genes is associated with c-onc activation [Lombardi et al., 1987; Wei et al., 1994]. In the present series, a weak correlation was found between the number of c-onc transcripts and the number of EBV transcripts in a given biopsy. All 5 biopsies expressing ≥6 c-onc specificities express at least 3 EBV-related genes, whereas 12 of 17 biopsies with ≤4 abundant c-onc transcripts express 2 or less EBV-related genes (Fig. 4). This might suggest that some c-onc activities might influence EBV expression. It should be noted that all 8 biopsies with abundant transcripts from 3 or more c-onc genes have high BARF0 expression, and only 2 BARF0 positive samples do not show raised c-onc expression. The number of different transcripts observed is dependent on the presence and amount of RNA transcribed, its half-life [Beelman and Parker, 1995], post-transcriptional events, the sensitivity of the method of detection and the proportion of tumor cells in the biopsy. We would expect that biopsies from later stage disease might contain a larger proportion of transformed cells giving rise to easier detection of rarer transcripts, but a concommitant increase in number of EBV genome equivalents per cell which would be expected to accompany this effect was not observed. The sensitivity of the ^{32}P-cDNA method used limits the detection of weakly expressed transcripts, but is useful for studies of biopsy samples because many tests may be undertaken on the very limited amount of RNA available.

Using a method capable of analyzing abundant transcripts from EBV-related genes and cellular proto-oncogenes, it was shown that gene expression is variable in biopsy specimens from naturally occurring tumors, and that the amount of excessive gene expression tends to increase with severity of disease. In contrast, no clear difference in the pattern of gene expression was seen in the unusual population of young North African NPC patients as compared to the more usual adult population.

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